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14. ABSTRACT: From our previously funded projects, we utilized 77 benign prostatic hyperplasia (BPH) and 96 prostate cancer (PC) samples that are pre-existing from African-American and Caucasian patients. Due to human subjects issues, the project is currently on hold since Dec 2005 and thus, results reported are based on the pre-existing samples and experiments performed during the first half of the grant year. The first hypothesis is under experimentation and we have performed immuno-histochemical analysis to determine CYP1B1 protein expression. Preliminary results demonstrate that the CYP1B1 protein is localized to the cytoplasm of PC cells. Additionally, the intensity of CYP1B1 staining is much higher in PC than BPH. In the second hypothesis, single nucleotide polymorphisms (SNPs) at three codons (119, 432, and 453) of CYP1B1 have been evaluated to determine if they are risk factors for racerelated PC. Racial differences are observed in allele frequencies as the variant at codons 119 ($P<0.05$) and 432 ($P<0.001$) are greater among Blacks whereas the 453 variant ($P<0.001$) is predominant in Whites. Within race, no differences were observed between BPH and PC at all SNP sites. Additionally, no differences were observed between stages (<T2c vs >T2c) and grades (<7 vs >7) of PC in either race. Clearance with human subjects protocol, continuation of SNP studies with additional samples to be collected; as well as further experimentation with aim #1 will be the focus in year 3.

15. SUBJECT TERMS
CYP1B1, Polymorphism, Prostate cancer, Race-related

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INTRODUCTION

Prostate cancer is the most frequently diagnosed malignancy and the second leading cause of death among men with cancer in the USA. When comparing races, the incidence and mortality rates of prostate cancer in African-Americans is higher than in Caucasians and Asians. Cytochrome P450 (CYP) 1B1 converts estrogens to the 4-hydroxy-catechol-estrogens. Studies show this catechol-estrogen to be mutagenic and may lead to prostate cancer. Polymorphisms of CYP1B1 have been associated with various types of cancers and recently, we have shown that CYP1B1 polymorphisms have higher risks for prostate cancer (Abstract; J. Urol. 171(Suppl. 4):111, 2004). However, such studies are lacking in race-related prostate cancer. There are at least 4 polymorphisms that have been identified on the CYP1B1 gene that results in a structural change in the enzyme and are at the following locations: codons 48 (C to G), 119 (G to T), 432 (C to G), and 453 (A to G). The main goal of this project is to investigate whether polymorphisms of the CYP1B1 gene can be a risk factor for race-related prostate cancer. To determine this, two specific aims are tested. In specific aim #1, the hypothesis that CYP1B1 gene is hyper-activated during malignant transformation of race-related prostate cells is tested. In the 2nd aim, the hypothesis that single nucleotide polymorphisms (SNPs) of the CYP1B1 gene have higher risk for race-related prostate cancer and correlate with hyper-activated CYP1B1 gene is tested. Data generated from these experiments will determine whether CYP1B1 gene expression differs between Caucasian and African-American prostate cancer samples. Also, these experiments will determine whether CYP1B1 SNPs are involved in race-related prostate cancer. This knowledge will help to understand the genetic basis for racial differences as well as identify the subjects who are at higher risk for prostate cancer.

BODY

Samples: Due to human subjects protocol issues, the current project has been on hold since Dec of 2005. Currently, we are working with the DOD HSRRB to have this resolved and progress has been made with the modified protocol approaching acceptance. From our previously funded projects, we have however, obtained a total of 77 benign prostatic hyperplasia (BPH) and 96 prostate cancer specimens that were pre-existing archival specimens from African-Americans and Caucasians. Thus experimental results are based on these samples and work performed during the first half of the grant year.

Task #1. To determine if the CYP1B1 gene is differentially expressed between races (African-Americans and Whites) and in different stages and grades of prostate cancer.

The procedure for immunostaining of CYP1B1 protein has been developed and evaluated in formalin-fixed, paraffin-embedded prostate specimens that have been sliced into 5 μm sections. The protocol utilizes a rabbit polyclonal antibody (1:300 dilution) against human CYP1B1 (BD Gentest Corporation, Woburn, MA). The staining procedure is based on a commercial kit from Santa Cruz Biotechnology (Santa Cruz, CA) and immunoperoxidase activity was developed with 3,3-diaminobenzidine. The sections were then counterstained with hematoxylin. Based on a few BPH (n=5) and cancerous (n=5) tissue sections evaluated, the CYP1B1 protein is determined to be localized to the cytoplasm of cancer cells. Protein expression was also observed in the smooth muscle cells of both BPH and prostate cancer specimens. Using Image J software to evaluate intensity of staining, it is apparent that the cancerous tissues stain much higher compared to BPH. In the upcoming year, we will evaluate more specimens to determine preciseness for protein expression.

The methodology to determine CYP1B1 RNA expression and enzyme activity will be developed and analyzed in the upcoming year.

Task #2. To determine if single nucleotide polymorphisms (SNPs) of the CYP1B1 gene are risk factors for the etiology of race-related prostate cancer and correlate with hyper-activity of its gene.

From the pre-existing BPH and prostate cancer obtained from African-American and Caucasian patients, DNA was collected by using a DNA extraction kit (Qiagen, Valencia, CA). Quantity and quality of DNA was measured at 260 nm and 280 nm by the use of a spectrophotometer. A two-step polymerase chain reaction (PCR) procedure was designed for the analysis of CYP1B1 polymorphisms. The primers of three of the polymorphic sites studied so far (codons 119, 432, and 453) and PCR conditions are summarized in Table 1. In the first PCR, DNA (10 ng) was amplified in a 20 ul reaction containing 1.5 mM $MgCl_2$, 0.8 mM dNTP mix, PCR buffer, and 0.5 units of Red-Taq polymerase (Sigma-Aldrich, St. Louis, MO), along with primer sets designed to contain the polymorphic sites (Table 1). In the sequence-specific PCR (SSP), each polymorphic fragment was further amplified under similar conditions as the first-step PCR except for the use of SSP primer sets (Table 1). Each of the SSP products were electrophoretically separated on 3% agarose gels using 180 volts at ambient temperature. The products were then visualized by ethidium bromide staining under UV light. To confirm genotyping, products of the first PCR were subjected to direct DNA sequencing. DNA was purified from gels using a QIAquick PCR purification kit (Qiagen; Valencia, CA). Sequence analysis of purified products was then determined by using the first PCR primers and ABI 377 Sequencer and Dye Terminator Cycle sequencing kit (Applied Biosystems Inc.; Foster City, CA). Confirmation of DNA sequence was done on at least 3 representative samples for each of the polymorphic types. Frequencies of the various genotypes and allele types of CYP1B1 polymorphisms in the different categories of samples were determined and tabulated. Chi-square analysis was used to test each of the polymorphisms for differences in genotypic and allelic frequencies between Whites and Blacks as well as between BPH and prostate cancer. Relative risk associated with a particular genotype or allele was estimated by calculating odds ratios (OR) along with 95% confidence intervals (CI).

Results to date of the genotypic and allelic frequencies of the three SNP sites of the CYP1B1 gene between African-Americans and Caucasians for both BPH and prostate cancer patients are shown in Tables 2 and 3, respectively. Interestingly among BPH patients, the variant genotype (G/G) and allele (G) at codon 432 are highly predominant in African-Americans as compared to Caucasians (Chi-square, $P<0.01$). OR (95% CI) were 15.13 (4.14-55.23) for the G/G genotype in Blacks compared to Whites. However, no differences in genotype or allele frequencies were observed at codons 119 and 453 ($P>0.05$). In prostate cancer patients on the other hand, although not significant by chi-square analysis, the codon 119 variant T/T is much more prevalent in Blacks compared to Whites with an OR (95% CI) of 3.19 (1.05-9.74) as compared to wildtype G/G. In concordance, allele frequency shows a significant difference as the 119 T is much higher in Blacks compared to Whites ($P<0.05$). Likewise, the other two codons (432 and 453) show a significant difference for both genotype and allele frequency between Blacks and Whites. The 432 variant G/G is predominant in Blacks compared to Whites with a OR (95% CI) of 13.69 (5.06-37.01) and the allele is significantly higher in Blacks ($P < 0.001$). At codon 453, interestingly the variant appears to play a protective role in Blacks compared to Whites as the G/G type is not observed based on 37 Black samples and the A/G heterozygous type displays a OR (95% CI) of 0.11 (0.04-0.30). Variant allele G is also significantly lower in Blacks compared to Whites, $P<0.001$.

When comparing between BPH and prostate cancer patients within races, no differences were observed between the diseases amongst either African-Americans or Caucasians for all SNP sites studied.

Prostate cancer samples were classified in terms of stage. Due to the small N size when divided between races, stage classifications were made as < T2c or $\geq$ T2c. Results for each race is shown in Table 4. No differences were observed between stages for either race although sample size is small. Likewise, cancer samples were classified in terms of pathological grade and were based as < 7 or $\geq$ 7. Table 5 shows results of samples based on grade for both Blacks and Whites and no differences were observed.

Table 1. First step PCR and SSP primers utilized to determine SNP in BPH and prostate cancer samples.

CODON 119

1st PCR

Primer	Sequence	Anneal Temp
C119-Rev	ccttccagtgctccgagtag	
C119-For	ccccatagtggtgctgaatg	47 C

SSP

Primer	Sequence	Anneal Temp
C119 G-Rev with C119-For	acggaaggaggcgaaggc	65 C
C119 T-Rev with C119-For	acggaaggaggcgaagga	65 C

CODON 432

1ST PCR

Primer	Sequence	Anneal Temp
C432-Rev	tcatcactctgctggtcagg	
C432-For	gtcttgggctaccacattcc	47 C

SSP

Primer	Sequence	Anneal Temp
C432 C-Rev with C432-For	tccgggttaggccacttcag	65 C
C432 G-Rev with C432-For	tccgggttaggccacttcac	65 C

CODON 453

1ST PCR

Primer	Sequence	Anneal Temp
C453-Rev	agaaagttcttcgccaatgc	
C453-For	gacccactgaagtggcctaa	47 C

SSP

Primer	Sequence	Anneal Temp
C453 A-Rev with C453-For	tctgctggtcaggtccttgt	64 C
C453 G-Rev with C453-For	tctgctggtcaggtccttgc	64 C

Table 2. Genotypic frequencies of CYP1B1 SNPs between races for BPH and Prostate cancer. P-value reflect chi-square test.

Type	Codon	Gene	White	Black	OR (95% CI)
BPH	119	G/G	21	16	Ref
		G/T	12	17	1.86 (0.71-4.88)
		T/T	6	5	1.09 (0.29-4.19)
	432	C/C	11	2	Ref
	P<0.01	C/G	20	14	3.85 (1.01-14.62)
		G/G	8	22	15.13 (4.14-55.23)
	453	A/A	28	33	Ref
		A/G	11	4	0.31 (0.10-0.95)
		G/G	0	1	-
PC	119	G/G	38	17	Ref
		G/T	14	10	1.60 (0.58-4.36)
		T/T	7	10	3.19 (1.05-9.74)
	432	C/C	23	3	Ref
	P<0.001	C/G	22	9	3.14 (0.88-11.14)
		G/G	14	25	13.69 (5.06-37.01)
	453	A/A	37	35	Ref
	P<0.01	A/G	19	2	0.11 (0.04-0.30)
		G/G	3	0	-

Table 3. Allele frequencies of CYP1B1 SNPs between races for BPH and Prostate cancer. P-value reflect chi-square test.

Type	Codon	Allele	White	Black
BPH	119	G	54	49
		T	24	27
	432	C	42	18
	P<0.001	G	36	58
	453	A	67	70
		G	11	6
PC	119	G	90	44
	P<0.05	T	28	30
	432	C	68	15
	P<0.001	G	50	59
	453	A	93	72
	P<0.001	G	25	2

Table 4. Genotypic frequencies of CYP1B1 SNPs between stages of cancer (< T2c vs ≥ T2c) for Blacks and Whites. P-value reflect chi-square test.

Race	Codon	Gene	< T2c	≥ T2c	P-value
Blacks	119	G/G	2	10	
		G/T	3	6	
		T/T	5	4	0.17
	432	C/C	2	1	
		C/G	2	5	
		G/G	6	14	0.43
	453	A/A	10	18	
		A/G	0	2	
		G/G	0	0	0.59
Whites	119	G/G	15	19	
		G/T	6	7	
		T/T	3	3	0.96
	432	C/C	12	9	
		C/G	8	13	
		G/G	4	7	0.37
	453	A/A	15	17	
		A/G	7	11	
		G/G	2	1	0.64

Table 5. Genotypic frequencies of CYP1B1 SNPs between grades of cancer (< 7 vs ≥ 7) for Blacks and Whites. P-value reflect chi-square test.

Race	**Codon**	**Gene**	**< 7**	**≥ 7**	**P-value**
Blacks	119	G/G	7	10	
		G/T	5	5	
		T/T	5	5	0.86
	432	C/C	1	2	
		C/G	5	4	
		G/G	11	14	0.76
	453	A/A	16	19	
		A/G	1	1	
		G/G	0	0	0.99
Whites	119	G/G	20	17	
		G/T	11	3	
		T/T	4	3	0.27
	432	C/C	14	9	
		C/G	15	7	
		G/G	6	7	0.44
	453	A/A	22	14	
		A/G	11	8	
		G/G	2	1	0.95

KEY RESEARCH ACCOPLISHMENTS:

- Established protocol to measure CYP1B1 in prostate tissue by immunohistochemistry.
- Evaluated CYP1B1 protein in BPH and prostate cancer samples.
- Established primers and protocol to measure three CYP1B1 SNPs.
- Evaluated CYP1B1 SNPs in BPH and prostate cancer samples.
- Evaluated CYP1B1 SNPs in stages and grade of prostate cancer samples.

REPORTABLE OUTCOMES:

Abstract presented at the American Association for Cancer Research (April, 2006).
The significances of the research performed to date are the following:
1) CYP1B1 protein is localized in the cytoplasm of prostate cancer cells with some expression in smooth muscle cells.
2) CYP1B1 protein has a much higher level in prostate cancer compared to BPH.
3) CYP1B1 codon 119 T variant genotype and allele are significantly higher in African-Americans with prostate cancer compared to Caucasians.
4) CYP1B1 codon 432 G variant genotype and allele frequency are significantly higher in African-Americans compared to Caucasians with BPH or prostate cancer.
5) CYP1B1 codon 453 G variant genotype and allele frequency are significantly lower in African-Americans compared to Caucasians with prostate cancer.
6) CYP1B1 polymorphisms did not correlate with stage or grade of prostate cancer.

CONCLUSIONS:

Racial differences in polymorphisms of the CYP1B1 gene exist and therefore, can identify the population with higher risk for prostate cancer.

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